PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day month year) 07 March 2001 (07.03.01)	
International application No. PCT/EP00/04855	**Applicant's or agent's file reference** G014WOORD019
International filing date (day/month/year) 27 May 2000 (27.05.00)	**Priority date** (day/month/year) 04 June 1999 (04.06.99)
Applicant KRAUSE, Steffi et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 December 2000 (15.12.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Olivia TEFY

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Copy for the Elected Office (EO/US)

PCT EP00 04855

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

GILHOLM, Stephen
Harrison Goddard Foote
31 St. Saviourgate
York YO1 8NQ
ROYAUME-UNI

Date of mailing (day month year)
18 January 2002 (18.01.02)

Applicant's or agent's file reference
G014WOORD019

IMPORTANT NOTIFICATION

International application No.
PCT/EP00/04855

International filing date (day month year)
27 May 2000 (27.05.00)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

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2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

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3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

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Form PCT/IB/306 (March 1994)

004601946

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
GO14WOORD01		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/04855	27/05/2000	04/06/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/00		
Applicant CAMBRIDGE LIFE SCIENCES PLC et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/2000	28.08.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Thumb, W Telephone No. +49 89 2399 7350

Form PCT IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/04855

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-10 as originally filed

Claims, No.:

1-17 as originally filed

Drawings, sheets:

1/5-5/5 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2-4, 10, 12-15
	No:	Claims	1, 5-9, 11, 16, 17
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-17
Industrial applicability (IA)	Yes:	Claims	1-17
	No:	Claims	

2. Citations and explanations
see separate sheet

Re Item II

Priority

Document SUMNER C. ET AL.: 'A transducer based on enzyme-induced degradation of thin polymer films monitored by surface plasmon resonance' ANAL. CHEM., vol. 72, 2000, pages 5225-5232, is not to be regarded as state of the art according to Rule 64.1 PCT as the date of priority claimed can be allowed for the relevant parts of the application

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   D1: SAUM A.G.E ET AL.: 'Use of substrate coated electrodes and AC impedance spectroscopy for the detection of enzyme activity' BIOSENSORS AND BIOELECTRONICS, vol. 13, 15 March 1998 (1998-03-15), pages 511-518.

   D2: US-A-5 846 744

   D3: HO W O ET AL: 'Electrochemical sensor for measurement of urea and creatinine in serum based on AC impedance measurement of enzyme-catalyzed polymer transformation' ANALYTICAL CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 71, no. 10, 15 May 1999 (1999-05-15), pages 1940-1946.

   D4: CHEN X. ET AL.: 'Dynamic surface events measured by simultaneous probe microscopy and surface plasmon resonance' ANAL. CHEM., vol. 68, no. 1996, pages 1451-1455.

2. Novelty

2.1 Document D1 describes the use of the catalytic activity of a protease to form the basis of an electrochemical sensor which detects the erosion of a surface layer of a protein by the enzyme (page 512, column 1, paragraph 4). Gelatine is coated on

the electrodes (page 513, section 2.3), and after immersion of the coated electrodes in a collagenase solution the change in impedance is measured at timed intervals (page 513, section 2.5). The AC impedance, measured with an alternating voltage or current, is a combination of both the resistive and capacitive properties of a material (page 511, column 2, lines 12-14). Differences in the concentrations of collagenase can be detected (page 515, figure 3). The detection of low concentrations of enzyme can be improved by stirring the solution (page 516, section 3.3).
Therefore, document D1 is novelty-destroying for the subject-matter of claims 1, 5-9, 11, 16, and 17 within the meaning of Article 33(2) PCT.

2.2 Document D2 describes a method which relies on the occurrence of an enzymatic reaction creating changes in the impedance of an electrode as a result of the partial or complete removal of an insulation polymer film from its surface. The enzyme can directly hydrolyse the polymer membrane and may be present in bulk solution (column 2, lines 41-57).
Document D2 is therefore detrimental to the novelty of claims 1, 5-9, and 16 (Article 33(2) PCT).

2.3 Dependent claims 2-4, 10, and 12-14 are novel in the sense of Article 33(2) PCT, as the therein referred to embodiments are not explicitly disclosed in the available state of the art.

2.4 Independent claim 15 is also formally novel as the modification of the method of claim 1 according to claim 15 is not explicitly disclosed in the state of the art.

3. Inventive step

3.1 Dependent claims 2-4, 10, and 12-14 do not contain any features which, in combination with the features of claim 1 to which they refer, meet the requirements of the PCT in respect of inventive step, the reasons being as follows:
Claims 2-4 and 10 refer to detection methods well known in the field of biosensors and therefore appear to be an obvious selection among a number of possibilities from which the skilled person would choose without the exercise of an inventive activity (see also PCT Guidelines, IV-8.8(C1)(i)). For example, document D3

discloses the detection of the transformation of a polymer coated onto carbon electrodes, said transformation being mediated by pH changes caused by enzymatic activity, using quartz crystal microbalance measurements (see page 1940, abstract). Document D4 describes the investigation of the degradation of a polymer surface via surface plasmon resonance (page 1453, column 1 and figure 2).

Claims 12-14 refer to specific embodiments of enzyme-substrate pairs. However, choosing an enzyme and a suitable substrate lies within the general knowledge of a person skilled in the art and cannot be considered as being inventive.

Claims 2-4, 10, and 12-14 therefore do not meet the requirements of Article 33(3) PCT.

3.2 Claim 15 differs from the teaching of document D1 (see item 2.1 above) in that a method for detecting enzymatic activity according to claims 1-14 is combined with a competitive immunoassay for detection of an analyte.

The underlying objective problem may therefore be seen in providing an alternative use of said method.

However, combining a method of detection with a standard immunoassay using competition between an enzyme-labelled analyte-analogue and the analyte is obvious for a skilled person and as such not sufficient to confer an inventive activity.

Claims 15 therefore does not comply with the provisions of Article 33(3) PCT.

METHOD AND APPARATUS FOR ENZYME DETECTION

Field of the Invention

The present invention relates to a method for detecting the presence of an enzyme, and an apparatus for use in the method.

Background of the Invention

Enzyme electrodes are well known in the art. For example, WO87/07295 and WO89/03871 disclose enzyme electrodes capable of responding amperometrically to the catalytic activity of the enzyme in the presence of its respective substrates, wherein the enzyme is immobilised or adsorbed onto the surface of an electrically conductive support member.

The advantages of amperometric biosensors which incorporate enzymes have been reviewed in some detail (Aston and Turner, Biotech. Genet. Eng. Rev. 1984, 1, 89-120, ed. G. Russell, Intercept, Newcastle-upon-Tyne; Davis G., Biosensors, 1985, 1, 161-178). The biosensors discussed therein vary in the mode of signal transduction and are loosely classified as (a) those in which the electrical response arises from the oxidation of a product of the enzyme reaction at an electrode, (b) "mediator assisted" reactions in which the electrons are transported from the enzyme to the electrode with the aid of an oxidation-reduction ("redox") reagent, or (c) "direct electron transfer (DET) in which no such mediator assistance is required.

There are several disadvantages associated with the use of a mediator in signal transduction, including the possibility of the mediator leaching out from the region containing the biocatalyst, diffusion limitations of oxidised and/or reduced forms, and the inherent instability of the mediator itself. As a consequence, mediatorless biosensors have been targeted as an alternative (Tarasevich, Bioelectrochemistry, 1985, 10, 231-295). Ianeillo et al (1982) Anal Chem 54, 1098-1101, describes mediatorless sensors in which glucose oxidase and L-amino acid oxidase were covalently bonded to a graphite electrode by the cyanuric chloride method. However, it was shown that these enzyme electrodes had only a limited working lifetime (Ianiello and Yacynynch, Anal Chem 1981, 53, 2090-2095).

Up to now, mediatorless enzyme electrodes have often incorporated conducting organic polymers, e.g. structural units similar to that of methyl viologen, and/or conducting organic salts such as $NMP^{+}TCNQ^{-}$ (N-methyl phenazinium tetracyano-4-quinodimethane) which modify the properties of the electrode and

fulfil the role of mediators. However, due to the instability of many conducting polymers, mediatorless electrodes of this type commonly exhibited a short half life and were often oxygen sensitive.

More recently, a novel sensor principle based on measurement of capacitance changes produced during enzyme catalysed dissolution of polymer coatings on electrodes has been developed (McNeil, C.J.; Athey, D.; Ball, M.; Ho, W.O.; Krause, S.; Armstrong, R.D.; Wright, J.D.; Rawson, K., Anal Chem 1995, 67, 3928-3935). Electrodes were coated with a biodegradable coating, a copolymer of methyl methacrylate and methacrylic acid. Dissolution is exemplified by a localised increase in pH near the surface of the coating due to the enzymatic reaction between urea and urease. Film degradation is accompanied by an increase in capacitance of up to four orders of magnitude. The method has been developed into a fast and simple disposable sensor for urea in serum and whole blood (Ho, W.O., Krause, S., McNeil, C.J., Pritchard, J.A., Armstrong, R.D., Athey, D. and Rawson, K. 1999 'Electrochemical sensor for measurement of urea and creatinine in serum based on AC impedance measurement of enzyme-catalyzed polymer breakdown'. Anal Chem In Press). Furthermore, it has been demonstrated that the high sensitivity and the fast response of this technique could be utilised for immunosensing using urease as the enzyme label.

However, there are several drawbacks associated with the above method, including the time required to produce the localised pH change to dissolve the polymer, the addition of an enzyme substrate and the need to wash (remove) excess enzyme label. Furthermore, following the polymer degradation by capacitance measurements only works effectively if the polymer coating is sufficiently insulating.

Thus, the present invention is advantageous as it addresses the aforementioned problems associated with the prior art.

Statements of Invention

In a broad aspect, the present invention provides sensors based on the enzyme-induced degradation of polymer films.

In one embodiment of the present invention there is provided a method for detecting the presence of an enzyme comprising contacting the sample to be analysed with a substrate, at least part of which is covered with a layer of a biodegradable polymer, said polymer being degraded by said enzyme to produce a signal; and measuring any signal produced.

In a preferred embodiment of the present invention the signal is measured by detecting changes in the polymer layer using quartz crystal microbalance.

In a second preferred embodiment of the invention the signal is measured by detecting changes in the polymer layer using surface plasmon resonance.

In a third preferred embodiment of the invention the signal is measured by detecting changes in the polymer layer using ellipsometry.

In a fourth preferred embodiment of the invention the signal is measured by detecting changes in the polymer layer using electrochemical impedance spectroscopy.

In a fifth preferred embodiment, the signal is measured by detecting changes in the polymer layer using capacitance measurements.

In one embodiment of the present invention the substrate is an electrode.

In a further embodiment of the invention, the substrate is a capacitor.

In another embodiment of the present invention the substrate is a transducer.

Preferably, the transducer is an electrochemical transducer, an optical transducer or a capacitor.

The enzymes of the present invention may be the analytes present in the sample or introduced as part of reagent system (e.g. an immunoassay label to detect an analyte present in the sample).

In one embodiment of the invention the biodegradable polymer is a poly (ester-amide) and the enzyme is a protease.

In another embodiment of the invention, the biodegradable polymer is a dextran hydrogel and the enzyme is a dextranase.

In another embodiment of the invention, the biodegradable polymer is an albumin crosslinked polyvinylpyrrolidone hydrogel and the enzyme is a pepsin

In another embodiment of the invention, the biodegradable polymer is a polyester such as poly (trimethylene succinate) and the enzyme is a lipase.

In a further embodiment the invention provides an assay comprising the steps of bringing a sample to be detected for the presence of an analyte into contact with a substrate comprising binding sites for the analyte, in the presence of a conjugate of the analyte and an enzyme label; and detecting the presence of unbound conjugate using the method of the present invention.

Preferably the samples used in the current invention are in the form of an aqueous sample, or a biological fluid, for example, blood, urine, serum, plasma or saliva.

In a further aspect, the present invention provides an apparatus for detecting the presence of an enzyme according to the method of any preceding claim comprising a substrate, at least part of which is covered with a biodegradable polymer.

Detailed Description of the Invention

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example with reference to the accompanying figures in which:

Fig. 1 shows the structure of poly(ester amide).

Fig. 2 shows SPR measurements showing degradation at different concentrations of α-chymotrypsin: (a) $4x\ 10^{-10}$M, (b) $4x\ 10^{-9}$M, (c) $9.6x\ 10^{-9}$M, (d) $2x\ 10^{-8}$M, (e) $2.8x\ 10^{-8}$M, (f) $4x\ 10^{-8}$M, (g) $1.2x\ 10^{-7}$M, (h) $2.8x\ 10^{-7}$M. (where 1 element = $5.3x10^{-3}$ degrees.)

Fig. 3 shows calibration curves for α-chymotrypsin assay for different molecular weights of poly(ester amide).

Fig. 4 shows impedance measurements during degradation of poly (trimethylene succinate) in the presence of lipase.

Fig. 5 shows SPR measurements during degradation of poly(trimethylene succinate) films at different concentrations of lipase from Pseudomonas fluorescens (42.5 U/mg).

In a preferred embodiment of the present invention, biodegradable polymer films are deposited onto the transducer surface of known thickness and are then dissolved (degraded) directly due to an enzyme or enzyme label acting on the polymer film. The enzyme or enzyme label is in close proximity or attached to the polymer film. The films proposed in the present sensor system are very homogeneous and respond in a matter of minutes due to enzyme amplification, thus resulting in higher sensitivities and lower limits of detection. Coating degradation may be followed using SPR, QCM or ellipsometry and the rate of dissolution of the film has been shown to be directly related to the concentration of enzyme.

Thus the present invention provides a new generic sensor format using biodegradable polymers such as poly (ester amides) which can be degraded specifically as a direct result of an enzymatic reaction. The major advantage over the prior art is that the electrode coating itself serves as the enzyme substrate, i.e. no additional enzyme substrate needs to be added or immobilised in order to mediate between the enzymatic reaction and the electrode coating. The present invention simplifies the sensor by reducing the number of sensor components and reactants as well as removing the absolute requirement for additional washing steps in the immunoassay. As a consequence, production costs should be reduced and the sensor systems should display increased reliability.

The signals measured in the present invention may be produced in response to a reduction of the polymer layer on the substrate, either in terms of the area of the substrate covered by the polymer layer, or in terms of the depth of the polymer layer. The signal may also be produced in response to the quality of the polymer layer, for example in terms of pore formation, swelling and/or delamination.

Quartz crystal microbalance (QCM), surface plasmon resonance (SPR) and ellipsometry may be used to determine properties of surfaces and thin films. All of these techniques have been applied successfully to biosensing, especially to monitoring of direct binding events between antigens and antibodies (Rickert, J,; Brecht, A.; Gopel, W., Biosensors and Bioelectronics 1997, 12, 567-575: Toyama, S.; Shoji, A.; Yoshida, Y.; Yamauchi, S.; Ikariyama, Y., Sensors and Actuators B-Chemical 1998, 52, 65-71: Arwin, H., Thin Solid Films 1998, 313-314, 764-774). Direct binding produces changes to the electrode surfaces that are more indicative of a porous layer, resulting in very small changes being observed.

Electrochemical impedance spectroscopy provides information about film properties such as incomplete coverage, pore formation, swelling and delamination. The initial film quality and film degradation of the present invention may be studied using electrochemical impedance spectroscopy over a frequency range from 0.1mHz to 100kHz. In addition to the information extracted from impedance spectroscopy, impedance measurements at quartz crystals provide data such as changes in mass and the visco-elastic properties of the films during degradation. In order to extract this information, the quartz-crystal impedance spectra may be fitted to the equivalent circuit of a coated quartz crystal given in Auge, J.; Hauptmann, P.; Eichelbaum, F.; Rosler, S., Sensors and Actuators B-Chemical 1994, 19, 518-522. Typically, impedance measurements are performed at polymer coated quartz crystals at a number of frequencies close to the resonance frequency of 10 MHz.

The term degradation is used in its conventional sense, i.e., a chemical reaction in which a compound is converted, or decomposes in some way, to give a simpler compound, for example, by dissolution. Monitoring film degradation using QCM, SPR, ellipsometry or electrochemical impedance spectroscopy has shown that the rate of dissolution of the polymer film is directly related to the enzyme concentration. Thus, using this system for the development of a generic immunosensor with enzyme as the anti-

gen and antibody label has the advantage that no enzyme substrate needs to be added. The electrode coating itself serves as the substrate thus making the electrode an integral part of the sensing process and eliminating washing steps otherwise required in standard immunoassay techniques.

The electrodes of the present invention are noble metal electrodes. Noble metals include metals such as gold, silver and platinum, or alloys thereof, which display resistance to corrosion or oxidation. Preferably the electrode is gold. Typically, the gold is deposited by thermal evaporation onto a chromium coated glass slide. The thickness of the gold coating may vary considerably, but is usually between 20 and 100 nm. Preferably, the thickness of the gold coating is between 45 and 80 nm.

The substrate is coated with a film of the biodegradable material. The film may range from monolayers to several hundred nm thick. Preferably, the film is from 5 to 100 nm thick. More preferably, the film is 10 to 100 nm thick. Typically, the films are deposited on the surface of the substrate by spin-coating using, a solution of the polymer in an appropriate solvent (for example, chloroform, or acetone). The biodegradable films degrade rapidly under the catalytic action of a specific enzyme directed to the polymer used to coat the substrate.

The preferred coatings in the present application degrade within a matter of seconds or minutes in the presence of a single enzyme, thereby leading to a fast sensor response. In contrast, up to now, most biodegradable materials described in the literature are reported to degrade over the course of several hours or days (Arabuli, N.; Tsitlanadze, G.; Edilashvili, L.; Kharadze, D.; Goguadze, T.; Beridze, V.; Gomurashvili, Z.; Katsarava, R., Macromolecular Chemistry and Physics 1994, 195, 2279-2289; Brondsted, H.; Hovgaard, L.; Simonsen, L., Stp Pharma Sciences 1995, 5, 60-64). Thus the present invention provides a sensor displaying short response times.

The assay of the present invention typically employs binding pairs. A non-exclusive list of commonly used binding pairs includes avidin/biotin, antibody/antigen, haptens and nucleic acid (DNA and RNA). Generally, when the binding pair is antibody/antigen the assay is referred to as an immunoassay. Other biosubstances capable of molecular recognition include lectins for saccharides, hormone receptors for hormones and drug receptors for drugs and active drug metabolites.

In a preferred aspect, the method of the present invention is used for performing an immunoassay.

Typically, in enzyme immunoassays, an enzyme is used as a label or marker which is bound to one member of the antigen-antibody pair identical to that in the sample to be measured. The enzyme bound antigen/antibody then competes with the sample antigen/antibody for the binding site on a limited supply of its complement antibody/antigen.

Classical methods for immunoassay include: (i) a capture antibody on a solid phase, such as a plastic microtitre plate, exposure to the biological sample to attach the antigen of interest, washing and then exposure to a second labelled antibody. The label on the antibody may be an enzyme for example. Further washing is followed by detection of the label (and hence the amount of antigen in the original sample). This is known as a sandwich assay or two-site assay. (ii) a capture antibody on the solid phase followed by exposure to the biological sample containing antigen and an added amount of labelled antigen. Labelled and unlabelled antigen compete on the solid phase for the antibody sites. The amount of label revealed after washing is inversely proportional to the amount of true antigen in the biological sample. This is known as a competitive assay.

The concept of integrating enzyme and immunoassay techniques into the sensor devices disclosed in the present invention thus offers the prospect of reagentless analysis with little or no sample preparation. The major advantage of this approach for medical use is ease of operation, thereby allowing deployment of sensors in decentralised laboratories and facilitating a more rapid return of clinical information. The net benefit is an earlier institution of appropriate therapy.

In a preferred embodiment an immunosensor can be produced where the sample flows through a series of zones. The first of these is a blood separation membrane, which removes the cellular component. In the next zone, the capture antibody or antigen is immobilised on a substrate such as nitrocellulose membrane or polystyrene. A sample is introduced containing the analyte to be measured and mixes with an enzyme/antigen or enzyme/antibody conjugate. The mixture of analyte and conjugate will then flow over the capture antibody or antigen. Both conjugate and analyte compete for the binding sites. Flow through the capture membrane will remove some of the enzyme-analyte conjugate in a competitive manner. In the next zone, the unbound complex reaches the biodegradable polymer and causes it to degrade. The rate of polymer dissolution is directly proportional to the amount of analyte in the sample. The immunoassay can be set up in the competitive or sandwich assay format.

The present invention is further illustrated by way of the following non-limiting examples.

Example 1

One such biodegradable material is poly (ester amide) shown in Figure 1. Poly (ester amides) of 18800 g mol^{-1}, 10218 g mol^{-1} and 6407 g mol^{-1} were synthesised by polycondensation as described in Arabuli, N.; Tsitlanadze, G.; Edilashvili, L.; Kharadze, D.; Goguadze, T.; Beridze, V.; Gomurashvili, Z.; Katsarava, R., Macromolecular Chemistry and Physics 1994, 195, 2279-2289. The polydispersity of the polymer was lowered to 1.28 using fractionation. 3 nm chromium and then 45 nm gold were thermally evaporated onto the whole surface of the glass slides. The shape is not critical as the laser beam hits the middle of the surface. The gold layer is on top of the chromium and therefore always in contact with the polymer. The two metals were deposited without opening the evaporator in between. 10 nm thick films of the poly (ester amides) were spin-coated onto the gold surface from a 0.13 w% solution of the polymer in chloroform at a speed of 3000 rpm. The polymer films were left to dry at room temperature for at least 24 hours. Electrochemical impedance spectroscopy and SPR were used to characterise the polymer films and to follow their degradation in the presence of α-chymotrypsin. The cell was thermostated at 25°C to eliminate any effect that temperature changes may cause in the rate of the polymer degradation. The detection system of the SPR monitor essentially consists of a monochromatic and polarised light source, a glass prism, a thin metal film in contact with the prism, and a photodetector.

The polymer was degraded rapidly by the proteolytic enzyme α-chymotrypsin. The rate of hydrolysis of esters is ~10^5 times higher than the corresponding amides when both are catalysed by α-chymotrypsin (Arabuli, N.; Tsitlanadze, G.; Edilashvili, L.; Kharadze, D.; Goguadze, T.; Beridze, V.; Gomurashvili, Z.; Katsarava, R., Macromolecular Chemistry and Physics 1994, 195, 2279-2289), i.e. α-chymotrypsin preferentially attacks the ester bonds. α-Chymotrypsin is also a suitable enzyme label for immunosensing since it is virtually never present in the blood circulation.

The polymer films were shown to be stable in a pH 7.3 buffer containing 140 mM NaCl and 10 mM phosphate. Addition of α-chymotrypsin to the buffer solution resulted in rapid and reproducible polymer breakdown. The degradation of the polymer was complete in less than 20 minutes for enzyme concentrations greater than 9x 10^{-9} mol/l (see Figure 2). After an initial period the SPR response changed linearly with time. To obtain a calibration graph, the slopes of the linear region of the breakdown curves in Figure 2 were calculated and the data presented in Figure 3. Using the rate of change as a measure for the enzyme concentration rather than the time needed to degrade the film completely has the advantage, that considerably lower enzyme concentrations can be detected in a reasonable period of time. However, to visualise the sensitivity over the whole concentration range, a logarithmic plot is shown (Figure 3). The calibration graph (Figure 3) shows that α-chymotrypsin concentrations as low as 4x 10^{-10} mol/l could be detected in less than 30 minutes. The degradation was shown to be dependent

ent on the enzyme concentration. Thus, the system can be used as part of an immunosensor based on the detection of α-chymotrypsin concentrations.

Example 2

Polyesters such as poly (trimethylene succinate) can be hydrolysed by lipases. The dissolution of poly (trimethylene succinate) powder and films was investigated by Walter, T.; Augusta, J.; Muller, R.J.; Widdecke, H.; Klein, J., Enzyme and Microbial Technology 1995, 17, 218-224. The enzyme activity for the interaction of lipase with an insoluble substrate was found to be highly reproducible. Succinic acid (38.8g, 0.33mol) and 1,3-propanediol (26.25mL, 0.33mol) were mixed in a flask under nitrogen with mechanical stirring and heated slowly to 90°C. Methane sulphonic acid (0.071g, 0.07mmol) was then added and the temperature raised to 100°C. Water vapour was evolved and collected using a micro-distillation head. The reaction was left overnight at 100°C then cooled to give a thick orange oil. Gel Permeation Chromatography (GPC) showed that the molecular weight was still very low so 28.0g of the pale orange oil was further reacted with (0.10g, 1mmol) of the catalyst overnight at around 100°C, the temperature was then increased to 140°C to remove any excess water. The polymer was then isolated by precipitation from methanol overnight to give a high molecular weight fraction of polymer. Total yield was 3g. GPC - Mw = 5765, Polydispersity = 1.212. Glass substrates were prepared from microscope slides and washed by boiling in 50:50 nitric acid (70%): hydrogen peroxide for 5 minutes. The slides were then washed with ultra pure water and dust free methanol before blow-drying with nitrogen. Chromium was then deposited to give a ~20nm layer, followed by an ~80nm layer of gold. Vacuum deposition was carried out with an Edwards E306A coating system, and an IL150 quartz crystal rate monitor was used to monitor the deposition rate and layer thickness. The gold-coated slides were spin-coated with the poly (trimethylene succinate) using acetone as the solvent and a concentration of 0.093g/mL of the polymer. As previously the electrodes were dried for 24h at room temperature before use. All the impedance measurements were performed using an Autolab frequency response analyser. Measurements were conducted at zero potential using a 2-electrode system with a platinum electrode as the counter electrode in parallel to the polymer coated gold electrode The polymer coated metallised glass slides were placed into the bottom of a Perspex well. The dimensions of the measuring area were determined by an O-ring with an inner diameter of 7 mm which formed the waterproof connection between the metallised glass slide and the well. A platinum coil was placed opposite the working electrode at a distance of ~4 mm and served as the counter electrode. The electrodes were characterised in 0.5mL of a pH 7.4 buffer solution containing 10mM phosphate and 100mM sodium chloride. The effect of the enzyme addition was investigated. Measurements were performed at a single frequency (3.5kHz) every 10s. 0.5mL of lipase solution (1mg/ml of lipase from Pseudomonas fluorescens with an activity of 42.5 U/mg in buffer) was added after around 20 min. The result (Figure 4) shows polymer degradation. The degradation of poly (trimethylene succinate) films in the presence of different concentrations of lipase was also followed using SPR measurements (Figure

5). Substrate preparation, spin coating and SPR measurements were carried out under the same conditions as described in Example 1.

Claims

1. A method for detecting the presence of an enzyme comprising contacting the sample to be analysed with a substrate, at least part of which is covered with a layer of a biodegradable polymer, said polymer being degraded by said enzyme to produce a signal; and measuring any signal produced.

2. A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using quartz crystal microbalance.

3. A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using surface plasmon resonance.

4. A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using ellipsometry.

5. A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using electrochemical impedance spectroscopy.

6. A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using capacitance measurements.

7. A method according to claim 1 wherein the substrate is a transducer.

8. A method according to claim 7 wherein the substrate is an electrode.

9. A method according to claim 7 wherein the substrate is a capacitor.

10. A method according to claim 7 wherein the transducer is an electrochemical transducer or an optical transducer.

11. A method according to any preceding claim wherein the biodegradable polymer is a poly (ester-amide) and the enzyme is a protease.

12. A method according to any one of claims 1 to 10 wherein the biodegradable polymer is a dextran hydrogel and the enzyme is a dextranase.

13. A method according to any one of claims 1 to 10 wherein the biodegradable polymer is an albumin crosslinked polyvinylpyrrolidone hydrogel and the enzyme is a pepsin.

14. A method according to any one of claims 1 to 10 wherein the biodegradable polymer is a polyester such as poly (trimethylene succinate) and the enzyme is a lipase.

15. An assay comprising the steps of bringing a sample to be detected for the presence of an analyte into contact with a substrate comprising binding sites for the analyte, in the presence of a conjugate of the analyte and an enzyme label; and detecting the presence of unbound conjugate using the method of any one of claims 1 to 14.

16. A method according to any preceding claim wherein the sample is an aqueous sample, or a biological fluid.

17. An apparatus for detecting the presence of an enzyme according to the method of any preceding claim comprising a substrate, at least part of which is covered with a biodegradable polymer.

Fig. 1

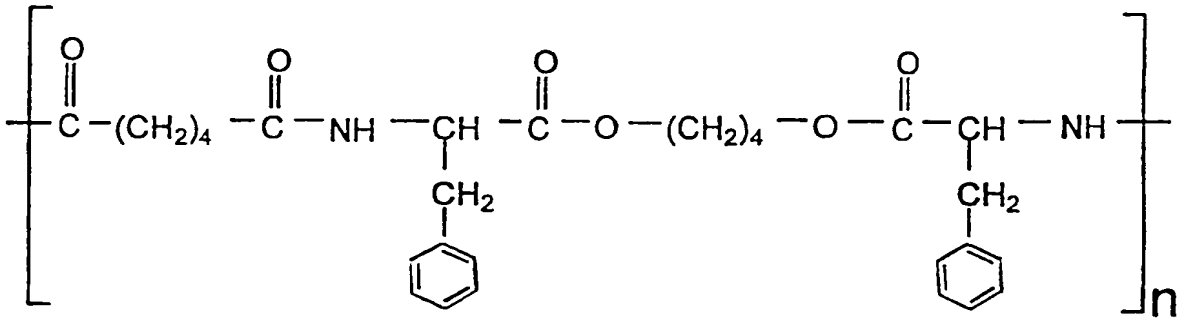
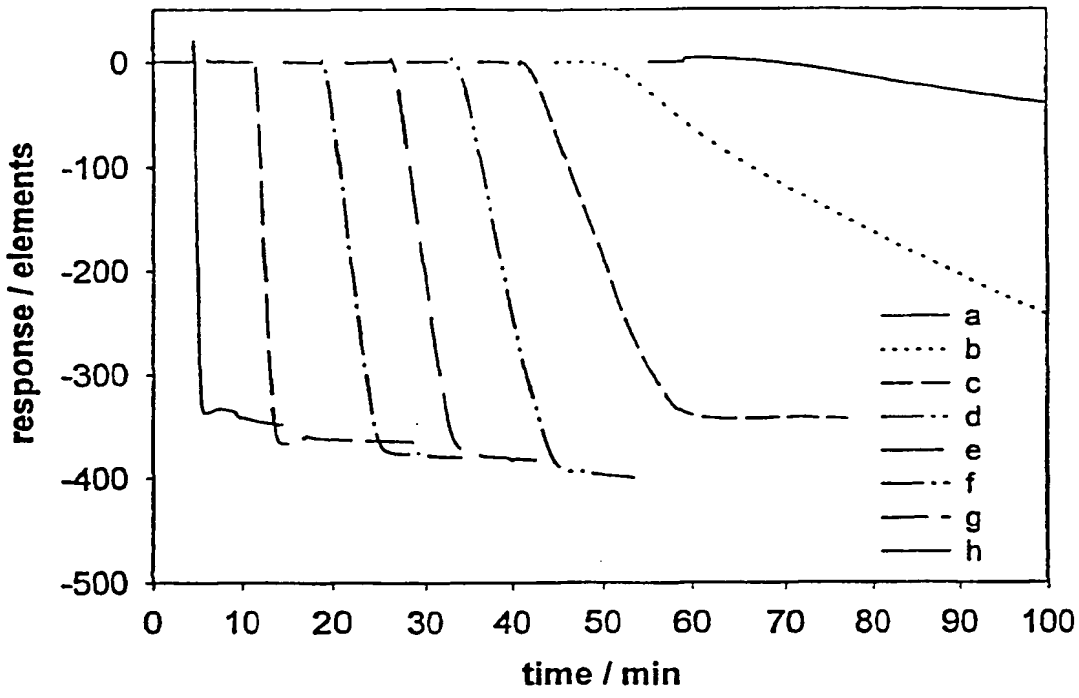


Fig. 2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a
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c
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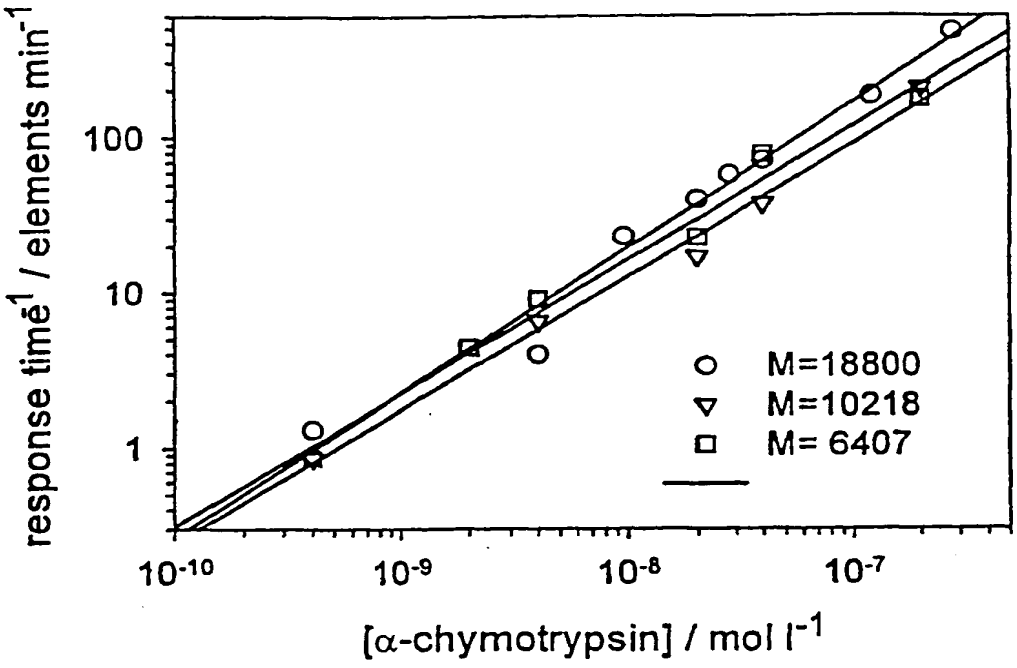
Fig. 3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10^{-10}
10^{-9}
10^{-8}
10^{-7}
[α-chymotrypsin] / mol l^{-1}
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M=10218
M= 6407

Fig. 4

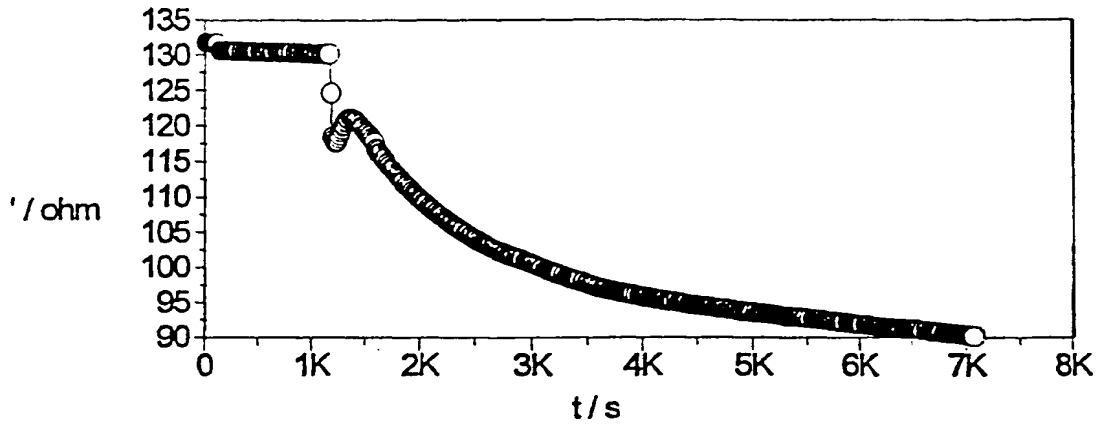
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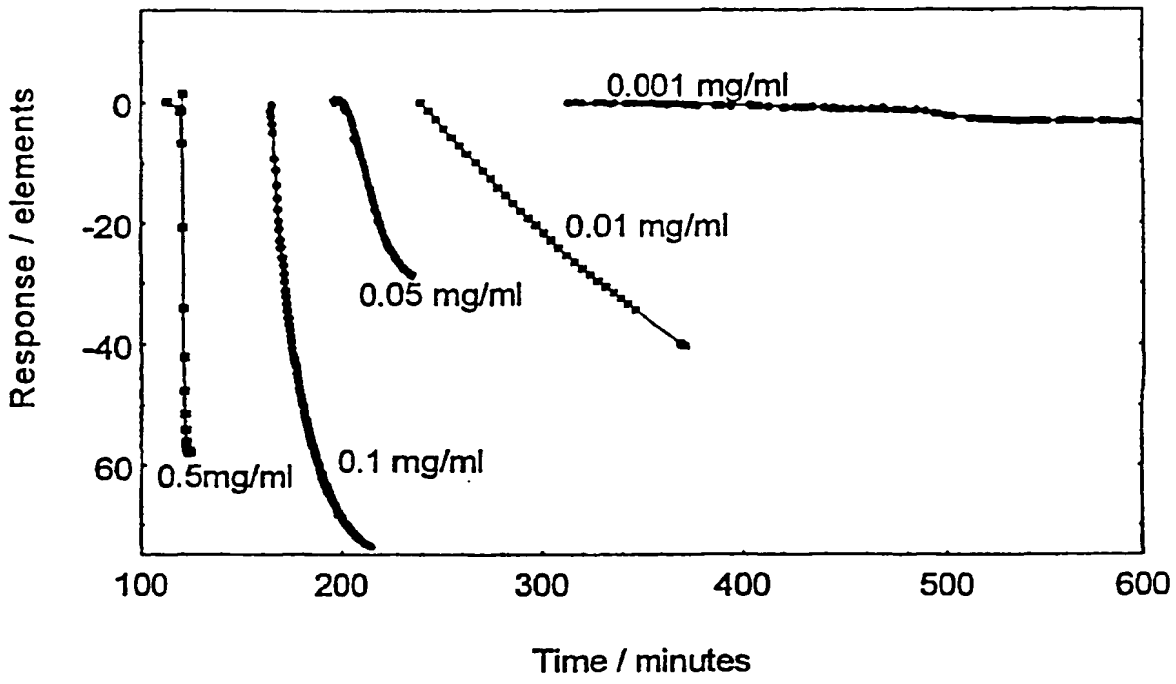
Fig. 5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400
500
600
Response / elements
Time / minutes

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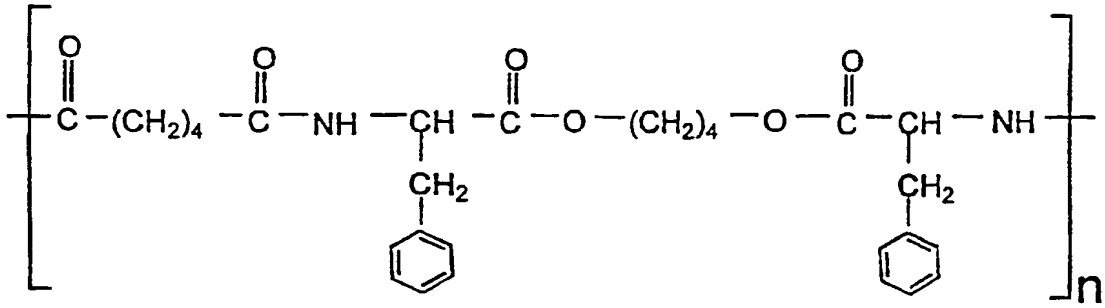
(54) Title: METHOD AND APPARATUS FOR ENZYME DETECTION



(57) Abstract: The present invention provides a method for detecting the presence of an enzyme based on the enzyme-induced degradation of a polymer film. In a preferred embodiment, the invention provides a method for detecting the presence of an enzyme comprising contacting the sample to be analysed with a substrate, at least part of which is covered with a layer of a biodegradable polymer, said polymer being degraded by said enzyme to produce a signal; and measuring any signal produced.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SUMNER C. ET AL.: "A transducer based on enzyme-induced degradation of thin polymer films monitored by surface plasmon resonance" ANAL. CHEM., vol. 72, 2000, pages 5225-5232, XP000961394 the whole document --- -/--	1-17

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Date of the actual completion of the international search	Date of mailing of the international search report
14 November 2000	18/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040. Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pellegrini, P

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G014W00RD019		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/04855	27/05/2000	04/06/1999
Applicant		
CAMBRIDGE LIFE SCIENCES PLC		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

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2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

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6. The figure of the **drawings** to be published with the abstract is Figure No. 1

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[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/04855

A. CLASSIFICATION OF SUBJECT MATTER
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Date of the actual completion of the international search	Date of mailing of the international search report
14 November 2000	18/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040 Tx. 31 651 epo nl Fax (+31-70) 340-3016	Pellegrini, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; AN PREV 199800360728, XP002152743 abstract & SAUM A.G.E ET AL.: "Use of substrate coated electrodes and AC impedance spectroscopy for the detection of enzyme activity" BIOSENSORS AND BIOELECTRONICS, vol. 13, 15 March 1998 (1998-03-15), pages 511-518, the whole document	1-17
X	US 5 846 744 A (ATHEY DALE ET AL) 8 December 1998 (1998-12-08) claims	1-17
X	HO W O ET AL: "ELECTROCHEMICAL SENSOR FOR MEASUREMENT OF UREA AND CREATININE IN SERUM BASED ON AC IMPEDANCE MEASUREMENT OF ENZYME-CATALYZED POLYMER TRANSFORMATION" ANALYTICAL CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 71, no. 10, 15 May 1999 (1999-05-15), pages 1940-1946, XP000834939 ISSN: 0003-2700 cited in the application the whole document	1-17
X	MCNEIL C.J. ET AL.: "Electrochemical sensors based on impendance measurement of enzyme-catalyzed polymer dissolution: theory and applications" ANAL. CHEM., vol. 67, 1995, pages 3928-3935, XP002151471 cited in the application the whole document	1-17
A	CHEN X. ET AL.: "Dynamic surface events measured by simultaneous probe microscopy and surface plasmon resonance" ANAL. CHEM., vol. 68, no. 1996, pages 1451-1455, XP002152742 the whole document	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter[illegible] Application No
PCT/EP 00/04855

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		GR	3025214 T	27-02-1998
		JP	8510833 T	12-11-1996
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		DK	700520 T	02-03-1998
		WO	9428414 A	08-12-1994
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